

Leptin improves intestinal flora dysfunction in mice with high-fat diet-induced obesity

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Abstract

Objective: This study investigated the effects of leptin on intestinal flora and inflammation in mice with high-fat diet (HFD)-induced obesity.

Methods: Mice were fed an HFD for 8 weeks; some were concurrently administered oral leptin for 4 weeks. Pathological changes in adipose tissue were detected using hematoxylin–eosin staining; endotoxin content in adipose tissue was measured by enzyme-linked immunosorbent assay. Intestinal flora were characterized by 16S bacterial rDNA sequencing. Levels of Toll-like receptor 4 (TLR4), nuclear factor- κ B inhibitor α ($I\kappa$ B- α), and phosphorylated c-Jun N-terminal kinase (p-JNK) were detected by western blotting.

Results: Mice in the HFD group exhibited weight gain, elevated endotoxin content, and adipocyte hypertrophy, compared with the non-obese control group. Moreover, abundance of bacteria in the *Bacteroides* genus and community diversity were both reduced in the HFD group; reductions also were observed at corresponding phylum, class, and order levels. Levels of TLR4, $I\kappa$ B- α , and p-JNK were also elevated in the HFD group. Compared with the model group, leptin administration reduced the weight gain and endotoxin content, while increasing *Bacteroides* abundance and community diversity; it also reduced the levels of TLR4, $I\kappa$ B- α , and p-JNK.

Conclusion: Leptin administration improved intestinal flora dysfunction and inflammation in mice with HFD-induced obesity.

Keywords

Leptin, intestinal flora, obesity, inflammation, high-fat diet, Toll-like receptor 4, *Bacteroides*, adipose tissue, nuclear factor- κ B inhibitor α , c-Jun N-terminal kinase

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Introduction

Obesity is characterized by weight gain and fat layer dysfunction.¹ The main reasons for obesity are excessive dietary intake and lack of physical activity, which lead to absolute or relative accumulation of energy.² The relationship between obesity and intestinal microflora has been gradually recognized.^{3,4} An imbalance in intestinal microflora structure causes an elevated number of opportunistic pathogens and pathogenic bacteria, thereby increasing the production of endotoxin; endotoxin and other adverse microbial metabolites affect gene expression and function in intestinal epithelial cells, which results in elevated intestinal mucosal barrier permeability.⁵ The accumulated toxins induce low-grade, chronic inflammation at the systemic level; this continuous inflammatory reaction is an important pathogenic factor that negatively affects individuals with obesity.⁶

Leptin is a bioactive protein with a molecular weight of 16 kDa. Its three-dimensional structure is similar to that of IL-6 family cytokines and consists of four α -helix bundles.⁷ Leptin is mainly secreted by adipocytes; it also exists in primary and secondary lymphoid organs. As an energy regulator, leptin controls satiety by stimulating the central nervous system and regulating the internal balance of glucose and insulin; these mechanisms have important metabolic and immune regulatory effects.⁸ Leptin is synthesized and secreted by adipocytes, which are mainly involved in regulation of food intake and consumption of energy.⁹ Leptin is presumed to be an anti-obesity hormone; thus, leptin therapy is proposed to prevent obesity and other diseases (e.g., hypertension and diabetes mellitus) through binding interactions with leptin receptors.¹⁰

The intestinal microflora are members of a complex ecosystem with more than 400 bacterial species. Recent studies have

shown that intestinal microflora dysfunction could contribute to a series of diseases,¹¹ especially obesity,¹² possibly through changes involving inflammation.¹² Notably, leptin has been reported to modulate intestinal microflora composition in mice.¹³ However, it remains unclear whether leptin regulates intestinal flora in high-fat diet (HFD)-induced obesity. In this study, the effects of leptin on intestinal flora and inflammation were investigated in mice with HFD-induced obesity. The results may be useful for understanding the anti-obesity activity of leptin.

Materials and methods

Animals and modeling protocol

Male C57BL/6 mice were purchased from Hunan Shrek Jingda Experimental Company (License No: scxk (Hunan) 2016-0002). This study was approved by the Ethics Committee of People's Hospital of Nanchang University (approval no. 2020081).

Forty-eight mice were randomly divided into four groups (n=12 mice per group): normal diet (control), HFD, HFD + 0.5 mg/kg leptin group (0.5 mg/kg), and HFD + 1.0 mg/kg leptin (1.0 mg/kg). Mice in the control group were fed a normal diet for 8 weeks; mice in the HFD group were fed an HFD for 8 weeks. In the 0.5 mg/kg and 1.0 mg/kg treatment groups, after 4 weeks of HFD consumption, leptin (cat. no. 25973, MedChemExpress) was orally administered once per day for the subsequent 4 weeks (0.5 mg/kg and 1.0 mg/kg, respectively). Mice were given free access to water and their designated diet. Their weights were also recorded. At the end of the 8-week experiment, all mice were anesthetized with isoflurane (5%) and decapitated. Adipose tissue and intestinal contents were collected and stored at -80°C for subsequent experiments.

Hematoxylin–eosin staining

Adipose tissues were fixed in 4% paraformaldehyde and dehydrated in a graded ethanol series, then embedded in paraffin and divided into 10- μ m-thick sections. Paraffin sections were baked, dewaxed, and rehydrated. They were then incubated in hematoxylin for 3 minutes and eosin for 3 minutes. After sections had been washed, they were dehydrated, sealed, and examined under a microscope (BX51, Olympus, Tokyo, Japan).

Endotoxin measurement

Endotoxin levels in adipose tissue were determined using an endotoxin assay kit (cat. no. MM-0369M1, MLBIO, Shanghai, China), as previously described.¹⁴ Results were expressed as endotoxin units per milliliter. All determinations were performed in a blinded manner.

Community diversity analysis

Genomic DNA was isolated using a Bacterial DNA Purification Kit (Merck, Kenilworth, NJ, USA), in accordance with the manufacturer's instructions. Amplicon libraries were prepared using a Nextera XT Index Kit (Illumina, San Diego, CA, USA). Before sequencing, library qualities were confirmed on an Agilent Bioanalyzer, using an Agilent High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA). DNA concentrations were then quantified on a QuantiFluor[®] RNA System using a PicoGreen dsDNA array kit (cat. no. P11496, Thermo Fisher Scientific, Waltham, MA, USA). Sequencing libraries were then diluted and mixed in accordance with the instructions of the MiSeq Reagent Kit v3 (600 cycles, Illumina). Sequencing of bacterial 16S rDNA was performed using a MiSeq sequencer for 2 \times 300 bp double terminal sequencing.

Western blotting

Total protein was extracted from the adipose tissue using a triplePrep kit (cat. no. 28-9425-44; ReadyPrep; GE Healthcare Life Sciences, Waukesha, WI, USA). Protein concentrations were determined using a bicinchoninic acid protein assay kit (cat. no. B9643, Sigma-Aldrich, St. Louis, MO, USA). Approximately 25 μ g/lane protein was separated by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes, as previously described.¹⁵ Membranes were blocked in 5% skim milk at room temperature for 2 hours, then incubated with the following primary antibodies overnight at 4°C: rabbit polyclonal anti-Toll-like receptor 4 (TLR4) (cat. no. bs-20594R; 1:100 dilution; Bioss, Woburn, MA, USA), rabbit polyclonal anti-phosphorylated c-Jun N-terminal kinase (p-JNK) (cat. no. ab76572; 1:5000 dilution; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-JNK (cat. no. ab4821; 1:5000 dilution; Abcam), and rabbit polyclonal anti-nuclear factor- κ B inhibitor α (I κ B- α) (cat. no. ab32518; 1:1000 dilution; Abcam). Membranes were then washed three times and incubated with secondary antibody (horseradish peroxidase-labeled goat anti-rabbit IgG; cat. no. A16104; 1:10000 dilution; Thermo Fisher Scientific) at 4°C for 2 hours. Blots were developed using an enhanced chemiluminescence kit (Thermo Fisher Scientific). Blots were imaged and analyzed using Quantity One software, version 4.62 (Bio-Rad, Hercules, CA, USA). Levels of p-JNK were normalized to the levels of JNK; levels of other proteins were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

All data were analyzed using SPSS Statistics, version 19.0 (IBM Corp.,

Armonk, NY, USA). Significant differences ($P < 0.05$) were determined by one-way analysis of variance with Bonferroni correction.

Results

Leptin administration reduces weight gain caused by HFD consumption

As shown in Figure 1, the weight of mice in the HFD group was significantly greater at the end of the 8-week experiment, compared with the weight of mice in the control group ($P < 0.05$). Leptin administration (0.5 mg/kg and 1.0 mg/kg) effectively reduced the weight gain caused by HFD consumption (both $P < 0.05$).

Leptin administration reduces endotoxin content associated with HFD consumption

As shown in Figure 2, the endotoxin content was higher in the HFD group than in the control group ($P < 0.05$). Leptin administration (0.5 mg/kg and 1.0 mg/kg) effectively

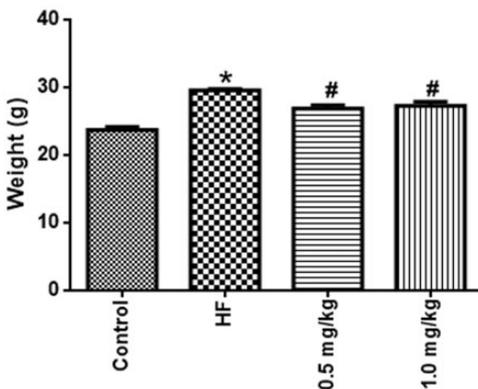


Figure 1. Leptin administration reduces weight gain caused by high-fat diet consumption. Error bars represent standard error of the mean. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. HFD group. Abbreviations: HFD, high-fat diet; 0.5 mg/kg, high-fat diet + 0.5 mg/kg leptin; 1.0 mg/kg, high-fat diet + 1.0 mg/kg leptin.

reduced the endotoxin contents, compared with the HFD group (both $P < 0.05$).

Leptin administration ameliorates adipocyte morphological changes caused by HFD consumption

As shown in Figure 3, mice in the HFD group exhibited remarkably hypertrophied adipocytes, with a substantially elevated cross-sectional area. In comparison with the HFD group, leptin administration (0.5 mg/kg and 1.0 mg/kg) improved the degree of adipocyte hypertrophy and reduced the cross-sectional area.

Leptin administration restores Bacteroides abundance in intestinal microflora altered by HFD consumption

Abundances of the phylum, class, order, and genus of *Bacteroides* bacteria were reduced by HFD consumption (all $P < 0.05$)

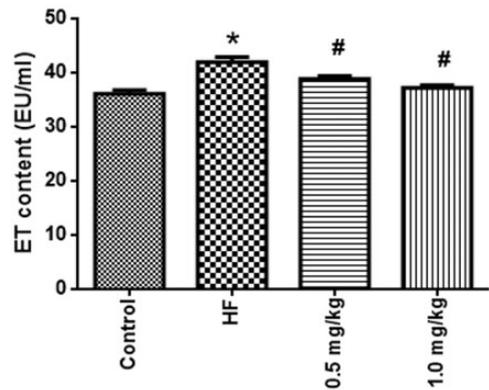


Figure 2. Leptin administration reduces endotoxin content associated with high-fat diet consumption. Error bars represent standard error of the mean. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. HFD group.

Abbreviations: HFD, high-fat diet; 0.5 mg/kg, high-fat diet + 0.5 mg/kg leptin; 1.0 mg/kg, high-fat diet + 1.0 mg/kg leptin; ET, endotoxin content; EU, endotoxin units.

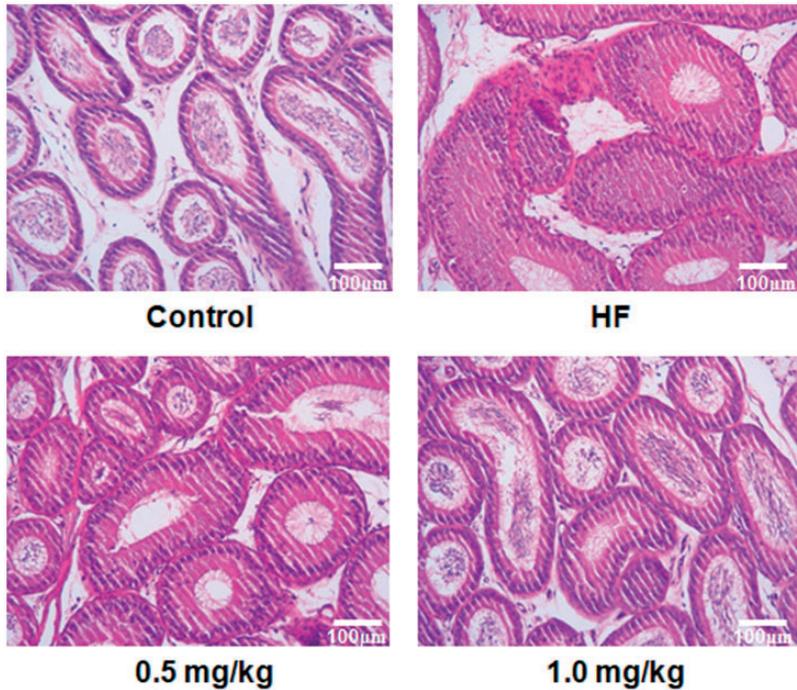


Figure 3. Leptin administration ameliorates adipocyte morphological changes induced by high-fat diet consumption. Scale bars = 100 μ m.

Abbreviations: HFD, high-fat diet; 0.5 mg/kg, high-fat diet + 0.5 mg/kg leptin; 1.0 mg/kg, high-fat diet + 1.0 mg/kg leptin.

(Figure 4a–d). In contrast, leptin administration (0.5 mg/kg and 1.0 mg/kg) significantly increased the abundances of these bacteria at all levels, compared with the HFD group (all $P < 0.05$) (Figure 4a–d).

Leptin administration restored alpha diversity in intestinal microflora altered by HFD consumption

All four groups exhibited similar abundance curves (Figure 5a). As shown in Figure 5b, there were no differences in the Simpson index among the groups. Compared with the control group, the Chao1, abundance-based coverage estimator, and Shannon indexes significantly decreased in the HFD group (all $P < 0.05$); these reductions were ameliorated by leptin

administration (Figure 5c–e) (all $P < 0.05$ vs. HFD group.).

Leptin administration ameliorated changes in TLR4, p-JNK, and I κ B- α expression caused by the HFD

Levels of TLR4, p-JNK, and I κ B- α were significantly higher in the HFD group than in the control group (all $P < 0.05$); these increased levels were all reduced by leptin administration (all $P < 0.05$ vs. HFD group) (Figure 6).

Discussion

In this study, we found that leptin administration reduced the weight gain caused by HFD consumption. Leptin also improved

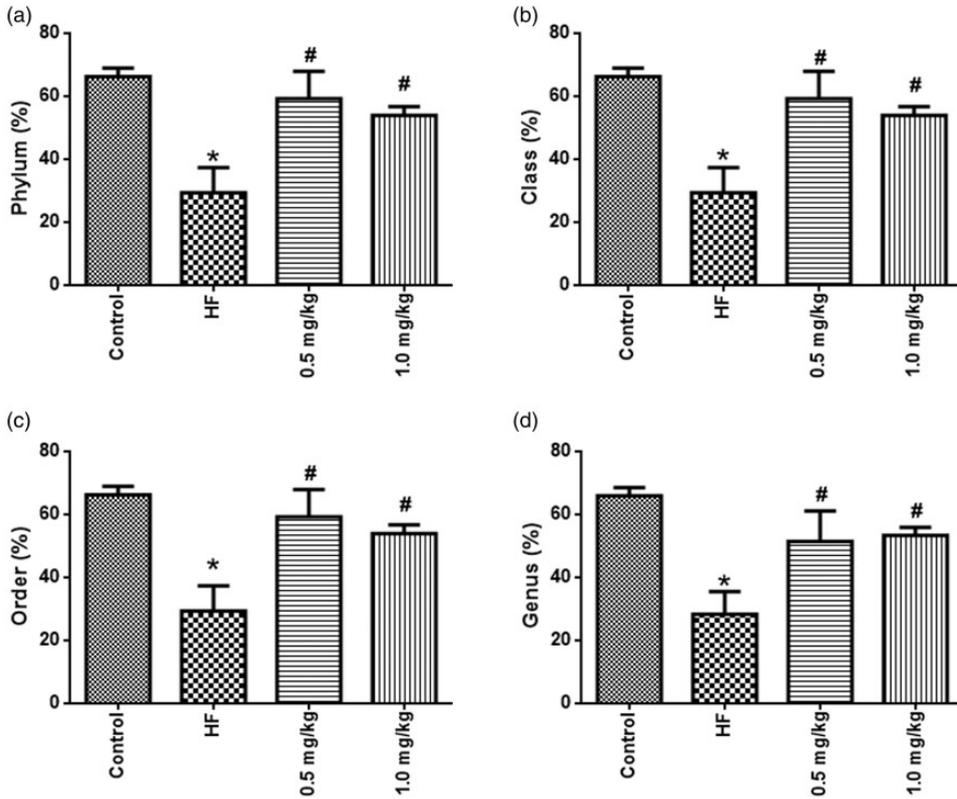


Figure 4. Leptin administration restores abundances of the phylum, class, order, and genus of *Bacteroides*, following reductions related to high-fat diet consumption. a) Phylum, b) Class, c) Order, d) Genus of *Bacteroides*. Error bars represent standard error of the mean. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. HFD group.

Abbreviations: HFD, high-fat diet; 0.5 mg/kg, high-fat diet + 0.5 mg/kg leptin; 1.0 mg/kg, high-fat diet + 1.0 mg/kg leptin.

gut flora dysfunction and reduced endotoxin content; biochemical analysis revealed that leptin reduced levels of TLR4, p-JNK, and $\text{I}\kappa\text{B-}\alpha$. These results indicated that leptin could modulate gut flora and inflammation in HFD-induced obesity.

Obesity is characterized by an imbalance in energy intake and expenditure; it is mainly modulated by genetic, environmental, and social factors.¹⁶ The incidence of obesity increases with economic development.¹⁷ Patients with obesity are likely to experience asthma and other comorbid diseases.¹⁸ In addition to physiological disorders,

obesity may cause psychological and social adaptation problems, such as reduced self-confidence, memory decline, mental retardation, and diminished social adaptability.¹⁹ Leptin in peripheral serum interacts with leptin receptors, potentially inhibiting appetite and increasing energy consumption.²⁰ In addition, leptin promotes the release of peripheral norepinephrine and activates adrenergic receptors on the adipocyte membrane by enhancing sympathetic activity; this process releases a large amount of stored energy as heat and increases energy consumption.²¹ Therefore, leptin plays an

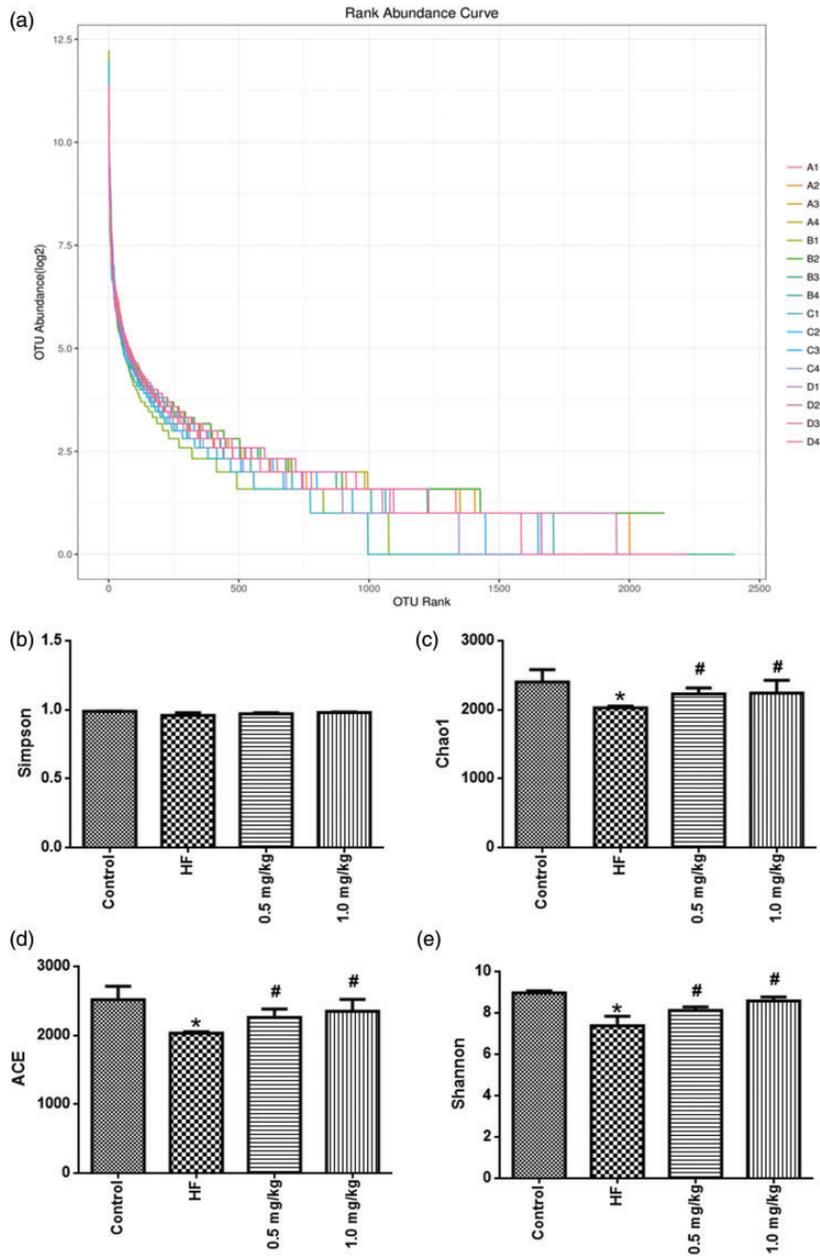


Figure 5. Leptin administration restores community diversity in intestinal microflora altered by high-fat diet consumption. a) Hierarchical abundance curves for each group. Length on horizontal axis reflects species richness. Shape of curve reflects species composition uniformity; b) Simpson index; c) Chao1 index; d) ACE index; e) Shannon index. Error bars represent standard error of the mean. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. HFD group. In panel a, letters denote indices (A, Simpson; B, Chao1; C, ACE; D, Shannon) and numbers denote groups (1, control; 2, HFD; 3, 0.5 mg/kg; 4, 1.0 mg/kg). Abbreviations: OTU, operational taxonomic unit; HFD, high-fat diet; 0.5 mg/kg, high-fat diet + 0.5 mg/kg leptin; 1.0 mg/kg, high-fat diet + 1.0 mg/kg leptin; ACE, abundance-based coverage estimator.

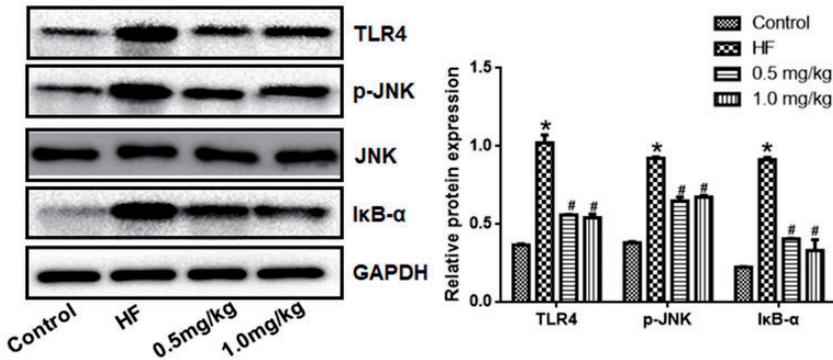


Figure 6. Leptin administration reduces TLR4, p-JNK, and IκB-α expression levels, following increases related to high-fat diet consumption. Error bars represent standard error of the mean. *P < 0.05 vs. control group; #P < 0.05 vs. HFD group.

Abbreviations: HFD, high-fat diet; 0.5 mg/kg, high-fat diet + 0.5 mg/kg leptin; 1.0 mg/kg, high-fat diet + 1.0 mg/kg leptin; TLR4, Toll-like receptor 4; p-JNK, phosphorylated c-Jun N-terminal kinase; IκB-α, nuclear factor-κB inhibitor α; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

important role in regulation of obesity.²² The results of this study revealed that leptin reduced the weight of mice who consumed an HFD; pathology analysis showed that leptin could improve the degree of adipocyte hypertrophy and reduce the adipocyte cross-sectional area.

Obesity is also a chronic inflammatory disease, characterized by elevated endotoxin content.²³ The level of endotoxin in the body is reportedly increased after HFD consumption.²⁴ Our study showed that endotoxin content in mice increased after HFD consumption, but decreased after leptin administration. Moreover, HFD consumption has been shown to cause changes in intestinal microflora, including bacteria without cell walls (i.e., Mollicutes).²⁵ In addition, the abundance and diversity of intestinal flora are reportedly negatively correlated with obesity.²⁶ Our results showed that the abundance of *Bacteroides* decreased; furthermore, obesity reduced the diversity of intestinal flora. The overall balance of the intestinal microflora is important in maintenance of homeostasis;²⁷ intestinal microflora are reportedly disordered in patients with obesity.²⁸ The results of the present study showed

that HFD consumption reduced the diversity of intestinal microflora in mice; however, leptin administration increased the diversity of intestinal microflora. Furthermore, the abundance of *Bacteroides* significantly decreased in the HFD group. Notably, administration of leptin led to changes in intestinal microflora community structure, as well as in the abundance of *Bacteroides*.

Continuous HFD consumption can induce obesity and increase expression levels of TLR4.²⁹ TLR4 is mainly activated by long-chain saturated fatty acids in animal models of HFD-induced obesity.³⁰ Notably, TLR4 regulates the expression of local cytokines, while promoting endoplasmic reticulum stress.³¹ Because TLR4 is a primary molecular target for saturated fatty acids, it may stimulate intracellular signaling that induces inflammation and alters appetite control.³² Our results showed that HFD consumption could increase TLR4 expression, while leptin administration could decrease TLR4 expression. These results suggest that leptin can reduce TLR4 expression in the context of obesity.

JNK is involved in many physiological and pathological processes, such as

embryo development, cell differentiation and apoptosis, and the immune response.³³ Inflammatory factors activate JNK activity in obesity.³⁴ The results of the present study showed that HFD consumption could increase p-JNK expression, while leptin administration could decrease p-JNK expression. Furthermore, I κ B- α is an important member of the I κ B family, which serves as a negative regulatory factor in the nuclear factor (NF)- κ B signaling pathway. By inhibiting the activation of NF- κ B, I κ B- α can terminate the inducible transcription of NF- κ B on inflammatory mediators.³⁵ I κ B- α regulates the rapid activation of NF- κ B.³⁶ When cells interact with specific stimuli, the I κ B kinase complex can promote phosphorylation and subsequent degradation of the I κ B- α protein. This degradation of I κ B- α allows the NF- κ B/Rel dimer to translocate from the cytoplasm to the nucleus, where it promotes the transcription of target inflammatory mediator genes.³⁷ Our results revealed that HFD consumption led to elevated levels of I κ B- α protein, whereas leptin administration led to reduced levels of I κ B- α protein.

In this study, we selected the TLR4 signaling pathway to represent inflammation because it plays an important role in initiating the innate immune response; notably, bacterial endotoxin-induced activation of TLR4 signaling is responsible for inflammatory disorders.³⁸ However, we acknowledge that additional proteins associated with the immune response and inflammation should be evaluated in future studies of the effects of leptin in obesity. Notably, the JNK and NF- κ B signaling pathways are two typical mediators of the immune response.³⁹ In future studies, we plan to use agonists and antagonists of these pathways to further investigate leptin-mediated changes in the context of obesity.

In conclusion, leptin administration led to improvement of obesity-related indicators, presumably by increasing the diversity

of intestinal microflora and improving the corresponding community structure, as well as by regulating the expression levels of the inflammation-related proteins TLR4, p-JNK, and I κ B- α .

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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